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Endogenous Retrotransposition Activates Oncogenic Pathways in Hepatocellular Carcinoma

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SUMMARY

LINE-1 (L1) retrotransposons are mobile genetic elements comprising ~17% of the human genome. New L1 insertions can profoundly alter gene function and cause disease, though their significance in cancer remains unclear. Here, we applied enhanced retrotransposon capture sequencing (RC-seq) to 19 hepatocellular carcinoma (HCC) genomes and elucidated two archetypal L1-mediated mechanisms enabling tumorigenesis. In the first example, 4/19 (21.1%) donors presented germline retrotransposition events in the tumor suppressor *mutated in colorectal cancers (MCC)*. *MCC* expression was ablated in each case, enabling oncogenic β -catenin/Wnt signaling. In the second example, suppression of tumorigenicity 18 (*ST18*) was activated by a tumor-specific L1 insertion. Experimental assays confirmed that the L1 interrupted a negative feedback loop by blocking *ST18* repression of its enhancer. *ST18* was also frequently amplified in HCC nodules from *Mdr2*^{-/-} mice, supporting its assignment as a candidate liver oncogene. These proof-of-principle results substantiate L1-mediated retrotransposition as an important etiological factor in HCC.

INTRODUCTION

Liver cancer accounts for 9% of all cancer deaths worldwide and 12% in developing countries (Jemal et al., 2011). Pathological inspection indicates hepatocellular carcinoma (HCC) in ~80% of liver tumors, with infection by hepatitis B virus (HBV) and hepatitis C virus (HCV) being the most prevalent risk factors, followed by chronic alcoholism (Jemal et al., 2011; Perz et al., 2006; Tateishi and Omata, 2012). Although early detection and monitoring of patients with liver cirrhosis can substantially improve 5 year survival rates, progression to advanced HCC reduces average life expectancy to less than 8 months (Llovet et al., 2008). As for other cancers, genome and exome resequencing have elucidated molecular pathways frequently perturbed in HCC (Guichard et al., 2012; Tateishi and Omata, 2012; Totoki et al., 2011), potentially enabling therapeutic intervention informed by the mutational signature of a given tumor. The capacity to catalog the full spectrum of genetic aberrations occurring in HCC is therefore of critical importance.

LINE-1 (L1) retrotransposons are a major source of endogenous mutagenesis in humans (Burns and Boeke, 2012; Levin and Moran, 2011). These mobile genetic elements utilize a “copy-and-paste” mechanism to retrotranspose to new genomic loci, with such success in germ cells that 500,000 L1 copies comprise ~17% of the genome (Lander et al., 2001). Of these copies, only 80–100 are transposition competent, with distinct subsets of frequently active—or “hot”—L1s driving insertional mutagenesis in each individual genome (Beck et al.,

2010; Brouha et al., 2003). Retrotransposon insertions can profoundly alter gene structure and expression (Cordaux and Batzer, 2009; Faulkner et al., 2009; Han et al., 2004; Levin and Moran, 2011) and have been found in nearly 100 cases of disease (Faulkner, 2011; Hancks and Kazazian, 2012). L1 activity is consequently suppressed in most somatic cells by methylation of a CpG island in the internal L1 promoter (Coufal et al., 2009; Swergold, 1990). By contrast, L1 is often hypomethylated in tumor cells, removing a key obstacle to retrotransposition (Levin and Moran, 2011).

Despite this failure to repress L1 transcription, only a handful of L1 insertions had been found in human tumors until very recently (Liu et al., 1997; Miki et al., 1992). High-throughput L1 integration site sequencing has since revealed 9 and 69 de novo L1 insertions, respectively, in lung and colorectal tumors (Iskow et al., 2010; Solyom et al., 2012), whereas cancer genome resequencing elucidated a further 183 tumor-specific L1 insertions in colorectal, ovarian, and prostate cancer (Lee et al., 2012). In this latter study, more than half of all insertions were found in a single colorectal tumor; the other individuals presented fewer than five tumor-specific L1 insertions on average. These data suggest L1 mobilization may be common in epithelial tumors, though the reasons for possible cell-of-origin restriction are currently unknown.

Tumor-specific L1 retrotransposition has not previously been observed in HCC. For several reasons it is, however, a logical cancer in which to expect L1 mobilization. First, HCC is epithelial in origin. Second, HBV and HCV infection are common in HCC; viruses can suppress host defense factors, such as APOBEC proteins, that control retrotransposon activation. APOBEC3G has been shown, for instance, to inhibit both HBV replication and endogenous retrotransposition (Esnault et al., 2005; Turelli et al., 2004). Third, liver inflammation precedes HCC and may, via cellular stress, stimulate retrotransposition (Fornace and Mitchell, 1986). Given these facts, we aimed to map L1 integration sites in HCC using retrotransposon capture sequencing (RC-seq) and assess their impact upon oncogenic and tumor suppressor pathways.

RESULTS

Enhanced Retrotransposon Capture Sequencing

To test the hypothesis that L1 mobilizes in HCC, we applied an updated RC-seq protocol to 19 HCC tumors and matched adjacent liver tissue that were confirmed positive for HBV or HCV infection (Table 1). An earlier RC-seq design (Baillie et al., 2011) was modified to incorporate multiplex liquid-phase sequence capture (Figure 1A) using a refined probe pool (Table S1 available online) and a reduced insert size of ~220 nt, which enabled high-confidence assembly of overlapping paired-end 150 nt reads (Figure 1B). This change simplified genomic alignment and, more importantly, enabled single-nucleotide resolution of retrotransposon integration sites (Figure 1C).

After stringent filtering and mapping, an average of ~2 million reads were retained per library with >95% identity to active L1, *Alu*, and SVA families, as well as the most recently active human LTR endogenous retroviruses (Table S2). Optimized sequence capture led to a 4-fold increase in reads aligned to nonreference

Table 1. Nonreference Genome Insertions Detected by RC-Seq

Donor	Gender	Virus	Age	Germline Insertions	Private Germline Insertions	Validated Tumor-Specific Insertions
12	M	HCV	65	2,082	202	3
15	M	HBV	53	1,845	216	1
21	M	HCV	51	2,019	271	0
29	M	HCV	52	1,602	44	0
32	M	HBV	73	1,681	100	0
33	F	HCV	57	1,982	234	2
35	F	HCV	78	1,786	96	0
42	F	HCV	67	1,594	43	0
47	M	HBV	61	1,581	77	2
48	M	HBV	35	1,744	212	0
49	M	HCV	68	1,644	58	0
60	M	HCV	48	1,570	33	0
62	M	HBV	33	1,750	153	0
70	M	HCV	55	1,673	82	0
86	F	HBV	56	1,701	50	0
89	M	HBV	60	1,739	163	4
95	M	HBV	54	1,773	88	0
106	M	HBV	60	2,141	48	0
116	M	HBV	62	1,532	71	0

F, female; M, male. Please see Tables S2 and S3 for supporting data and details.

genome L1s per library compared to previous RC-seq based on solid-phase arrays and similar sequencing depth (Baillie et al., 2011). The improved resolution of RC-seq also allowed us to discriminate a required minimum of two unique amplicons in support of any nonreference genome insertion (see Extended Experimental Procedures).

Frequent Retrotransposition in the Human Germline

A total of 7,689 nonreference genome insertions were detected in 19 tumor (T) samples and 19 matched nontumor (NT) liver samples. Of these, we annotated 7,644 as putatively germline (Table S3) because of their presence in (1) databases of retrotransposon-induced polymorphisms (Beck et al., 2010; Ewing and Kazazian, 2010; Iskow et al., 2010; Wang et al., 2006), (2) pre-existing insertions annotated by pooled blood RC-seq (Baillie et al., 2011), (3) multiple individuals, or (4) nontumor liver. L1, *Alu*, SVA, and LTR-flanked retrotransposons comprised 13.5%, 81.8%, 4.3%, and 0.4% of germline insertions, respectively. As expected, L1-Ta and L1-pre-Ta (99.3%) and *AluY* (99.7%) were the main L1 and *Alu* subfamilies active in germ cells (Mills et al., 2007).

A total of 2,241 germline insertions were found in only one individual each (Table 1 and Table S3) and were not annotated by the aforementioned retrotransposon polymorphism databases, suggesting that these were private or rare mutations or, alternatively, had occurred in early development (Garcia-Perez et al., 2007; Kano et al., 2009). RC-seq detected 1,489 (66.4%) insertions at both their 5' and 3' ends, enabling us to model the characteristic sequence features of L1-mediated retrotransposition.

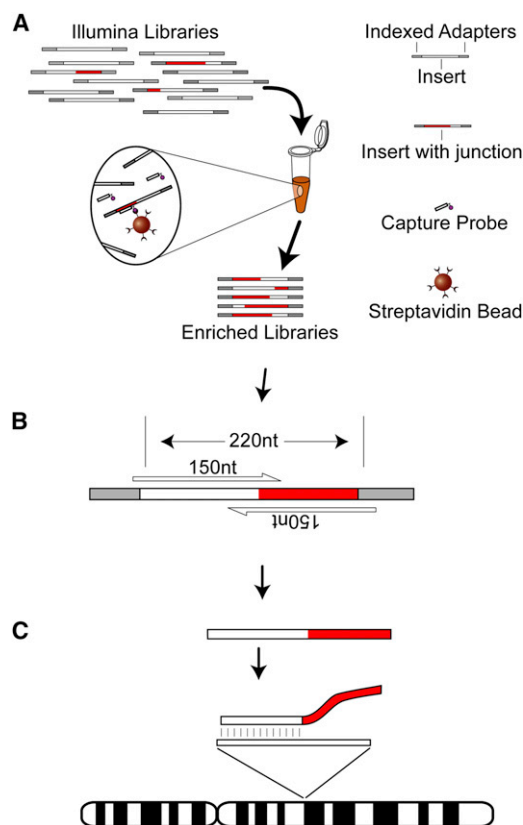


Figure 1. Enhanced RC-Seq

(A) Multiplexed Illumina libraries are hybridized to liquid-phase sequence capture probes targeting the 5' and 3' ends of recently active human retrotransposons (Table S1).

(B) Paired-end 150-mer sequencing of ~220 nt inserts enables "contig" assembly of each read pair into a single read.

(C) Assembled reads with a 5' or 3' section of an active retrotransposon at one end (highlighted in red) are retained. The opposite end is then aligned to the reference genome, indicating the position of known and novel insertions.

Without any additional sequencing, we were able to analyze insertions for the presence of target site duplications (TSDs), an L1-endonuclease recognition motif (Jurka, 1997), and a polyA tail (Figures 2A and 2B). These features consistently resembled target-primed reverse transcription (TPRT) for L1, *Alu*, and SVA, again illustrating the primary retrotransposition mechanism in germ cells (Cost et al., 2002; Jurka, 1997).

We also identified 160 previously undetected full-length (>99.9%) L1 copies, including 115 with paired 5'/3' detection (Figure 2C; Table S4) and 82 each found in a single donor only. All were annotated as L1-Ta or pre-Ta. These potentially "hot" L1s added to a recent cohort of full-length L1 insertions found in six geographically diverse individuals via fosmid screening and sequencing (Beck et al., 2010). Of 68 L1 insertions reported by Beck et al. (2010), we detected 49 (72.1%), including 15/18 (83.3%) with an allelic frequency >5%. Of the 49 insertions common to both studies, 46 (93.9%) were base-pair identical in genomic position. These results confirm strong agreement between RC-seq and the conservative fosmid-based approach of Beck et al. (2010).

Each individual genome contained on average 244 nonreference genome L1 insertions, a figure 60% and 80% higher, respectively, than recent L1 insertion site sequencing on cell lines (Ewing and Kazazian, 2010) and single cells (Evrony et al., 2012). Therefore, to assess the RC-seq false-positive rate, we randomly selected 200 germline insertions (173 *Alu*, 14 L1, 11 SVA, and 2 LTR) for site-specific PCR validation (Table S5). Of these, we confirmed 197 (98.5%). The remaining three insertions (2 SVA and 1 *Alu*) occurred in repetitive genomic regions and were detected by multiple unique reads in at least ten different samples each, indicating that these may have represented PCR false negatives. These comparisons and experiments together demonstrate the sensitive and accurate mapping of bona fide retrotransposition events by RC-seq and further highlight ongoing L1 retrotransposition in the global human population (Beck et al., 2010; Ewing and Kazazian, 2010; Huang et al., 2010; Iskow et al., 2010).

Activation of β -Catenin/Wnt Signaling via L1-Mediated Ablation of *MCC*

To assess the potential tumorigenic consequences of the identified nonreference genome insertions, we selected and validated, by insertion site PCR, 31 L1, *Alu*, and SVA insertions in genes generally implicated to play a causal role in cancer (Futreal et al., 2004) or specifically in HCC (Guichard et al., 2012), including L1 insertions in the proto-oncogene *ALK* and the tumor suppressor *FHIT* (Table S5). Quantitative RT-PCR indicated, however, that 28/31 of these germline insertions did not significantly perturb host gene expression in tumor or nontumor liver versus control liver from five unaffected individuals (data not shown).

Strikingly, the three remaining insertions all coincided with strong inhibition of the tumor suppressor mutated in colorectal cancers (*MCC*) (Higgins et al., 2007). *MCC* is expressed in liver (Senda et al., 1999) and regulates the oncogenic β -catenin/Wnt signaling pathway frequently activated in HCC (Fukuyama et al., 2008; Guichard et al., 2012; Totoki et al., 2011). In vitro experiments have established that siRNA knockdown of *MCC* mRNA dramatically increases β -catenin (CTNNB1) expression, whereas *MCC* overexpression inhibits cellular proliferation (Fukuyama et al., 2008; Matsumine et al., 1996). *MCC* is also an intriguing HCC candidate gene because of its genomic proximity to *APC*, a major tumor suppressor mutated in familial adenomatous polyposis preceding colorectal cancer (Grodin et al., 1991; Kinzler et al., 1991). It is important to note that mutated *APC* occurs in <2% of HCC cases versus >60% of colorectal carcinomas (Guichard et al., 2012; Powell et al., 1992). We therefore hypothesized that germline retrotransposition events specifically inhibited *MCC* tumor suppressor function in liver. To test this prediction, we assessed the impact of each *MCC* mutation upon *MCC*, *APC*, and *CTNNB1* expression.

Three germline retrotransposon insertions were found in *MCC*. The first of these, labeled *MCC*-L1- α , comprised a 5.3 kb L1-Ta oriented in sense to *MCC* in donors 70 and 95 (Figure 3A). Another L1-Ta, labeled *MCC*-L1- β , was full-length (6 kb), occurred at a different genomic position in donor 116, and was oriented antisense to *MCC* (Figure 3B). Finally, in donor 33, we found an *AluY* (*MCC*-*Alu*; Figure 3C) inserted in an

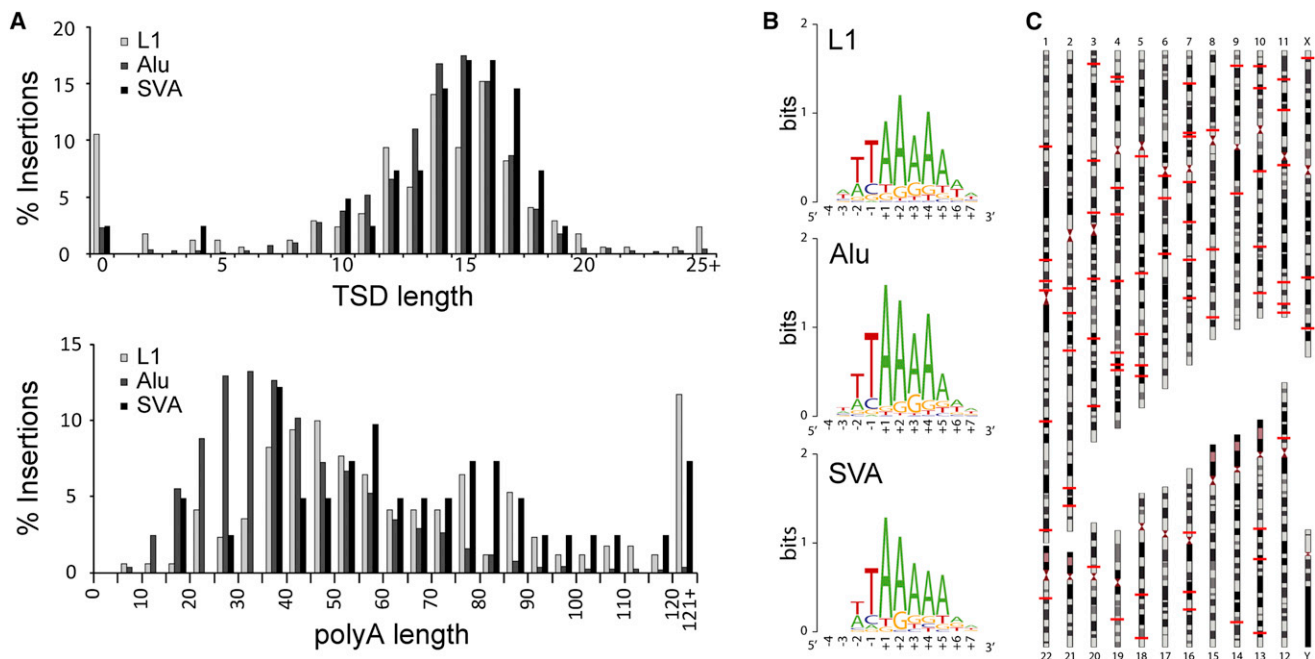


Figure 2. Characteristics of Recent Germline Retrotransposition in Humans

(A) Distributions of target site duplication length and poly-A tail length for L1, Alu, and SVA.

(B) Consensus sequence motifs (Crooks et al., 2004) at integration sites closely resembled the canonical L1 endonuclease recognition sequence.

(C) Genomic positions (indicated by red lines) of 115 previously unobserved full-length L1 insertions detected at both termini by RC-seq.

Please see Tables S4 and S5 for further supporting data.

ENCODE-delineated enhancer (Thurman et al., 2012). Insertion site PCR revealed that *MCC-L1- α* was heterozygous in donor 70 and homozygous (or possibly hemizygous) in donor 95, whereas *MCC-L1- β* and *MCC-Alu* were heterozygous in donor 116 and donor 33, respectively (Figure 3D).

An immunoblot indicated that MCC was dramatically less abundant in tumor and nontumor samples from all four donors compared with control liver tissue (Figure 4A). By contrast, CTNNB1 was expressed much more strongly in the affected donors than in controls (Figure 4A). This inverse relationship was consistent with MCC suppression of CTNNB1 through protein-protein interactions, as reported elsewhere (Fukuyama et al., 2008). As a corroborating example, immunohistochemistry performed on tumor and nontumor tissue from donor 116 confirmed cytoplasmic CTNNB1 accumulation (Figure S1), a strong indicator that the factors controlling CTNNB1 expression outside of the plasma membrane were absent and that many cells had entered a proliferative state (Nhieu et al., 1999).

Quantitative RT-PCR indicated that MCC transcription was severely reduced ($p < 0.02$ – $p < 0.002$, t test, degrees of freedom [df] = 19) in all four tumors compared to normal liver (Figures 4B). *MCC-L1- α* and *MCC-L1- β* strongly suppressed MCC expression in donor 95 and donor 116's nontumor liver, respectively (Figure 4B). MCC was also significantly downregulated in tumor versus nontumor in all four individuals ($p < 0.0001$, t test, df = 4). Capillary sequencing of each MCC exon revealed only one missense mutation, a homozygous SNP (570 A > G) in donor 33 MCC exon 5, producing an Arg > Lys substitution in the putative CTNNB1 binding domain of MCC (Fukuyama et al., 2008).

Therefore, *MCC-L1- α* , *MCC-L1- β* , and *MCC-Alu* were the primary enactors of MCC transcriptional inhibition, potentially assisted by other modifications to MCC or its upstream regulatory pathway.

Finally, we performed qRT-PCR to evaluate APC transcription coincident with mutated MCC. We found no significant differential transcription of APC in tumor or nontumor liver from the four affected donors versus normal liver controls (Figure S2). In donor 95, APC was downregulated significantly in tumor versus nontumor ($p < 0.003$, t test, df = 4) but only by 30% versus normal liver controls. By contrast, *MCC-L1- α* , the homozygous L1 insertion in donor 95, severely reduced MCC transcription in both tumor (–83%) and nontumor (–63%) samples compared with normal liver controls (Figure 4B), demonstrating that the primary effect of *MCC-L1- α* was on MCC rather than APC. These data in sum confirmed that (1) L1-mediated retrotransposition in MCC specifically repressed MCC and not APC and (2) CTNNB1 was strongly induced in all four affected individuals, indicating activation of a major HCC oncogenic pathway.

Somatic L1 Mobilization in HCC

Forty-five nonreference genome insertions were annotated as tumor specific. These consisted of 17 L1, 27 Alu, and 1 SVA. We first validated each L1 insertion with insertion site PCR, including capillary sequencing of their 5' and 3' ends (Table S6). All 17 L1s successfully amplified; 12 confirmed as tumor-specific, and 5 were found in both tumor and nontumor liver. Further examination of the tumor-specific set revealed uniform usage of the degenerate L1 endonuclease motif highlighted in Figure 2B. In

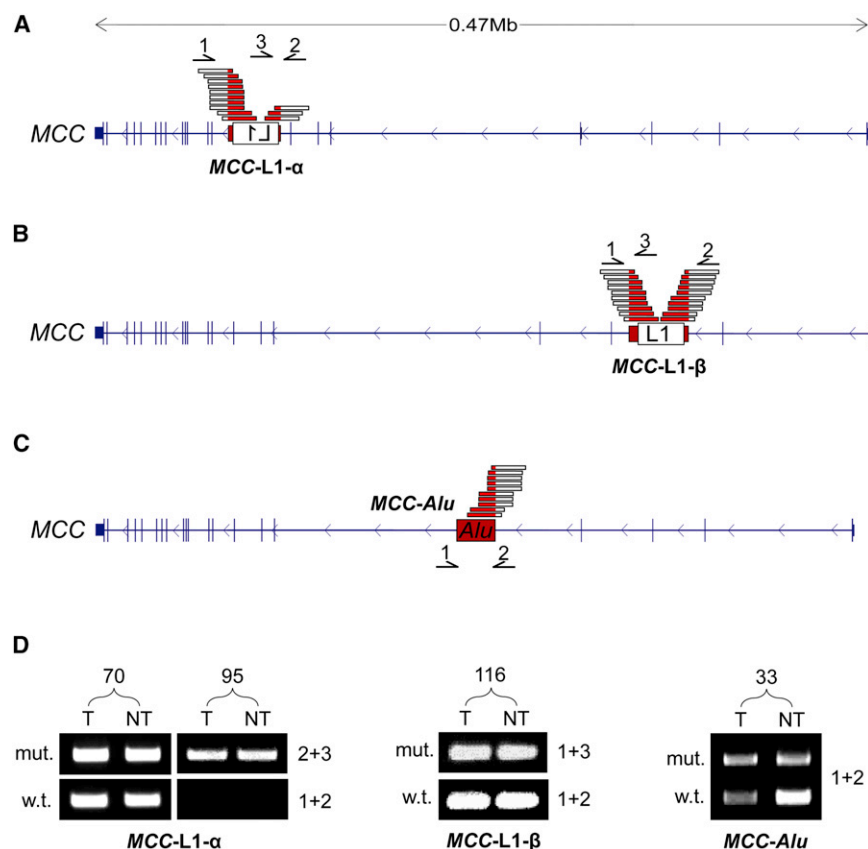


Figure 3. Structure and Validation of Germline L1 and Alu Insertions in MCC

(A) MCC mutant allele *MCC-L1-α*: a 5.3 kb L1-Ta detected by RC-seq at its 5' and 3' ends in 70T, 70NT, 95T, and 95NT. The L1 was flanked by a 13 nt TSD. Primers used for PCR validation (1,2,3) and RC-seq reads (red/white bars) are indicated above the gene structure. Note: L1 not drawn to scale.

(B) MCC mutant allele *MCC-L1-β*: a full-length (6 kb) L1-Ta detected by RC-seq at its 5' and 3' ends in 116T and 116NT. The L1 was antisense to *MCC* and was flanked by a 14 nt TSD. Primers are indicated as for (A).

(C) MCC mutant allele *MCC-Alu*: an AluY detected by RC-seq at its 3' end in 33T and 33NT. The AluY was antisense to *MCC*, had a 15 nt TSD, and bisected an annotated enhancer (Thurman et al., 2012). Primers (1,2) are indicated below the gene structure.

(D) Insertion-site PCR validation confirmed that *MCC-L1-α*, *MCC-L1-β*, and *MCC-Alu* were present in the corresponding tumor and nontumor samples (Table S5). The wild-type allele was absent for donor 95, indicating a homozygous L1 insertion.

two examples, PCR amplification of the 5' junction was repeatedly unsuccessful, preventing TSD characterization, an outcome possibly due to gross genomic abnormality at the L1 insertion site (Gilbert et al., 2002). Eight of the other integration sites incorporated TSDs, whereas the remaining two examples involved small genomic deletions 3' of the insertion site and no TSD. Somatic L1 mobilization occurred in donors 12, 15, 33, 47, and 89 (Table 1), with the latter individual presenting four insertions. Two L1 copies (chr11:60136439 and chrX:99180431) were greater than 5.3 kb in length, but no insertions were full-length. All 12 somatic L1 insertions were from the L1-Ta subfamily.

We next evaluated 13 *Alu* insertions and the single SVA insertion found only in tumor, using insertion site PCR. In all cases, amplification occurred in both tumor and adjacent liver DNA, indicating germline insertions. Our primary explanation for this result is that there are several thousand potentially active AluY copies in the genome, compared to fewer than 100 active L1s (Bennett et al., 2008; Brouha et al., 2003). As seen previously, the RC-seq read count per *Alu* is consequently 75% lower than for L1 (Baillie et al., 2011), making false-negatives in the nontumor control more likely for *Alu* than for L1. A secondary explanation is that chromosomal gain is very common in HCC (Guichard et al., 2012), increasing the probability that some germline insertions are detected in tumor but not in adjacent nontumor liver. A final possibility is that mutations in individual precancerous cells are clonally amplified in tumors and are called as tumor-specific by RC-seq and germline by insertion

site PCR. However, this was unlikely, as we consistently observed strong PCR amplification in both tumor and nontumor liver in these cases. Consequently, RC-seq reliably identifies new L1, *Alu*, and

SVA mobilization events but requires insertion site PCR to annotate tumor-specific insertions.

In recent work, we reported somatic L1 mobilization in the normal brain but did not evaluate other organs (Baillie et al., 2011). For the current study, somatic L1 insertions in nontumor liver were considered difficult to evaluate because of the frequent occurrence of chromosomal loss in tumors. In this scenario, germline L1 insertions may be deleted in tumor but retained in nontumor liver and called somatic events. Nonetheless, we identified 21 L1 insertions restricted to nontumor liver in the set putatively annotated as germline and as a proof-of-principle experiment selected an example (chr13:27423763) for insertion site PCR and capillary sequencing (Table S6). This 2.5 kb L1-Ta insertion was detected only in liver and, interestingly, had a long (127 nt) TSD (Figure S3). A germline L1 insertion deleted in tumor cells would reasonably be expected to be detected in the nontransformed cells (e.g., lymphocytes) infiltrating a tumor (Unitt et al., 2005). Therefore, this very likely represented a bona fide liver-specific somatic L1 insertion in the preneoplastic liver of donor 47. Consequently, hepatocytes, or their progenitor cells, may support limited somatic L1 mobilization, though the contribution of this activity to malignancy remains unclear.

L1 Hypomethylation Enables Tumor-Specific Mobilization

To assess whether L1 activity and L1 methylation state were correlated in HCC samples, we performed bisulphite conversion

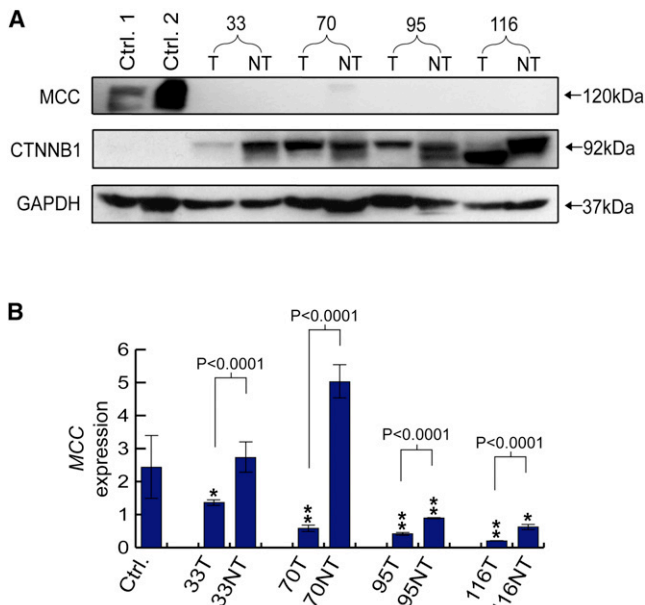


Figure 4. Downregulation of MCC

(A) Relative expression of MCC and CTNNB1 in control liver tissue compared to tumor and nontumor liver tissue from donors 33, 70, 95, and 116. An immunoblot performed against anti-MCC, anti-CTNNB1, and anti-GAPDH (loading control) antibodies detected strong MCC expression only in controls and strong CTNNB1 expression only in MCC mutant donors. MCC was also detected weakly in donor 70NT, in line with qRT-PCR results shown in (B). Expected protein molecular weights are marked on right. Note: anti-MCC and anti-CTNNB1 antibodies produce double bands. See Figure S1 for donor 116 CTNNB1 immunohistochemistry.

(B) Downregulation of MCC transcription in MCC mutant donors: qRT-PCR revealed that MCC mRNA was significantly reduced compared to control liver tissue in donors 33 (tumor only), 70 (tumor only), 95 (tumor and nontumor), and 116 (tumor and nontumor). $**p < 0.002$ and $*p < 0.02$, two-tailed t test, $df = 19$. In all four donors, MCC was also strongly downregulated in tumor versus nontumor samples ($p < 0.0001$, two-tailed t test, $df = 10$). Data are presented as mean \pm SD. See Figure S2 for APC qRT-PCR.

of gDNA and capillary sequenced the CpG island present in the canonical L1 promoter. Eight tumors (15T, 47T, 48T, 62T, 89T, 95T, 106T, and 116T) matched adjacent liver samples, and control liver samples were analyzed. In the tumor group, 54.8% of L1-promoter CpG dinucleotides were methylated, compared with 69.2% in nontumor liver, a strongly significant difference ($p < 2.5 \times 10^{-18}$, chi-square test, $n = 8$) (Figure 5A). On average, all but one CpG was hypomethylated in tumor, with the remaining CpG being equally methylated in tumor and nontumor liver (Figure S4A). Hypomethylation was not observed in grouped adjacent nontumor liver tissue versus controls.

As shown in Figure S4B, a subset of four individuals (donors 47, 89, 106, and 116) presented much stronger L1-promoter hypomethylation in their tumor (40.5%) versus nontumor liver (72.3%) samples compared with the remaining individuals (69.2% versus 66.1%). The three individuals with tumor-specific L1 insertions and L1 methylation data (donors 15, 47, and 89) yielded a strong correlation between L1 hypomethylation percentage and tumor-specific L1 insertion count ($r = 0.97$; $n = 3$). Donor 89 exhibited the strongest tumor-specific L1 hypomethy-

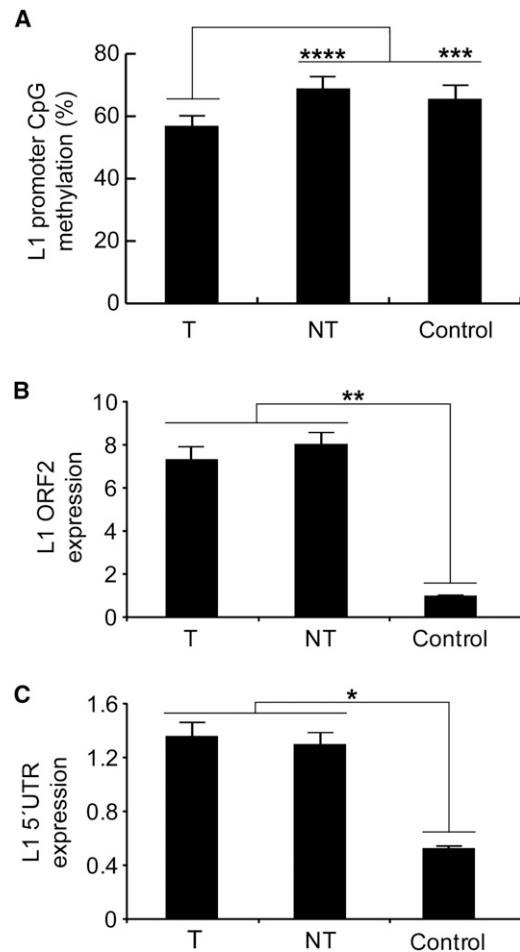


Figure 5. L1 Promoter Activation in HCC

(A) Bisulphite analyses in HCC patients versus controls revealed a significant decrease in L1 promoter methylation in tumor samples. Each column represents the methylation of 20 CpG residues found within the internal L1-Ta promoter. Values are presented as the mean percent of CpG methylation \pm SEM ($***p < 0.0005$, $****p < 2.5 \times 10^{-18}$, chi-square test). Please see Figure S4 for detailed analysis.

(B) TaqMan qRT-PCR measurement of L1 ORF2 indicated significantly increased L1 transcription in tumor and adjacent matched liver tissue versus controls. Data for each group (tumor, nontumor, and control) were pooled and presented as mean \pm SEM ($**p < 0.003$, two-tailed t test, $df = 22$, Bonferroni correction).

(C) As for (B), except observed at the L1 5' UTR ($*p < 0.006$). Please see Figure S3 for an example of a somatic L1 insertion in nontumor liver.

lation and also had the most tumor-specific L1 insertions (Figure S4C). Donor 15 showed only tumor-specific hypomethylation distal to the L1 5' end, whereas donors 47 and 89 were hypomethylated across the L1 promoter (Figure S4C).

Hypomethylation of the L1 promoter enables transcription of full-length L1 mRNAs that are translated to form the L1 mobilization machinery (Ostertag and Kazazian, 2001a). We therefore used cDNA synthesized with L1-specific primers (Wissing et al., 2012) to quantify L1 expression levels by TaqMan qRT-PCR. In this analysis, we measured L1 mRNA levels using primers targeting L1 ORF2 (Figure 5B) and the L1 5' UTR (Figure 5C). In both

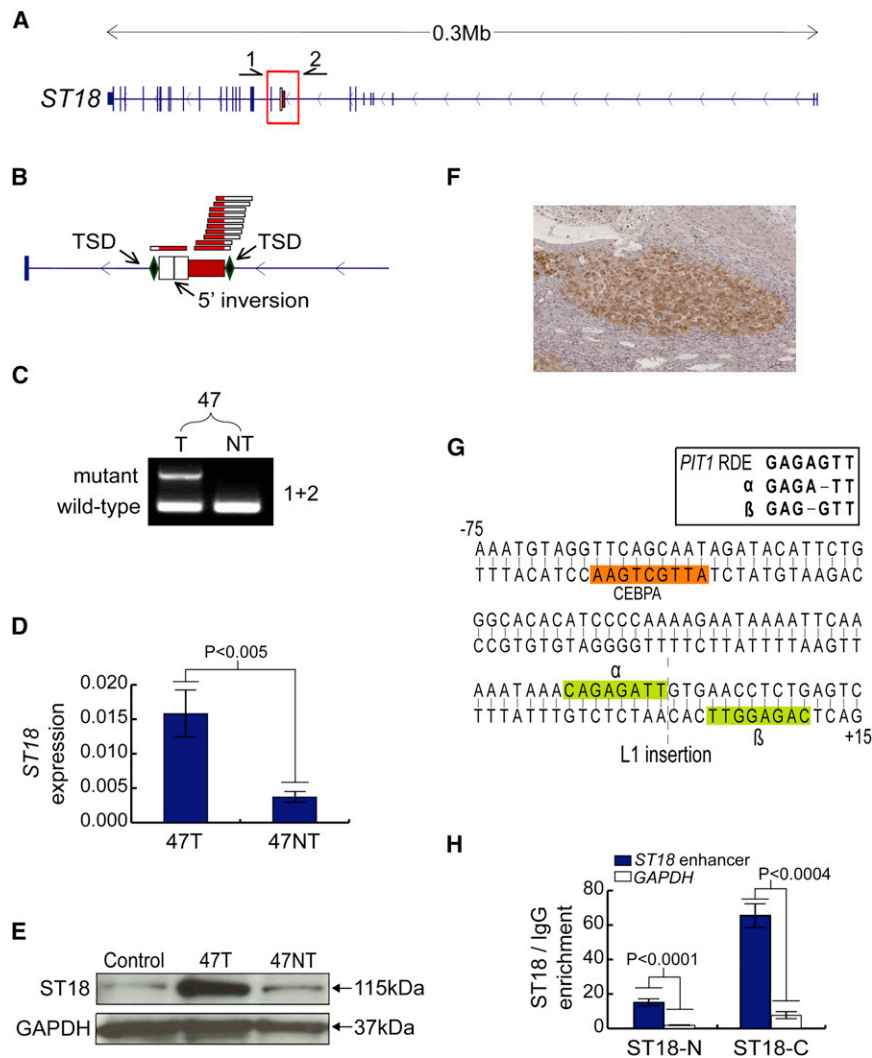


Figure 6. A Tumor-Specific L1 Insertion Causes Induction of *ST18*

(A) *ST18* mutant allele: a 0.4 kb L1-Ta insertion antisense to *ST18*. Primers used for PCR validation (1,2) are indicated above the gene.

(B) L1 insertion, magnified view: RC-seq detected the L1 5' and 3' termini, indicating a 17 nt TSD and a 5' inversion.

(C) Insertion-site PCR validation: the L1 was detected only in 47T, whereas the empty site was found in both 47T and 47NT.

(D) qRT-PCR: *ST18* was upregulated 4-fold in 47T versus 47NT ($p < 0.005$, two-tailed t test, $df = 4$). Data are presented as mean \pm SD.

(E) *ST18* immunoblot: *ST18* (115 kDa) was enriched in 47T versus 47NT and normal liver controls.

(F) *ST18* immunohistochemistry: accumulation of *ST18* (brown) was observed in tumor nodules compared to surrounding nontumor regions. Nuclei were stained with hematoxylin (blue).

(G) A palindromic sequence motif was bisected by the L1. Each 8 nt unit (α and β , light green) contained a subsequence 1 nt different to a *PIT1*-enhancer motif known to bind MYT1 (Rhodes et al., 1993). A second motif -58 bp from the L1 integration site matched the consensus CEBPA binding motif (orange).

(H) ChIP followed by quantitative real-time PCR in Huh7 cells confirmed enrichment for *ST18* bound to the putative *ST18*-enhancer element illustrated in (G), compared to GAPDH. Data from antibodies targeting both the N termini and C termini of *ST18* are shown. Significance values were calculated using two-tailed t tests ($df = 4$). Data are presented as mean \pm SD.

Please see Tables S6 and S7 and Figures S5 and S6 for further information regarding tumor-specific L1 insertions and additional *ST18* characterization.

cases, significant enrichment was observed in tumor and non-tumor versus normal controls ($p < 0.003$ for ORF2, $p < 0.006$ for 5' UTR, t tests, $df = 22$). Together, these data showed that L1 was activated and transcribed in HCC, coincident with hypomethylation of the L1 promoter.

***ST18* Activated by a Tumor-Specific L1 Insertion**

Tumor-specific L1 insertions were observed in six protein-coding genes (Table S6). Quantitative RT-PCR indicated that two of these genes (*STXBP5L* and *SLC5A8*) were not expressed in liver. The expression of three other genes was reduced 2-fold to 6-fold in tumor versus adjacent liver ($p < 0.05$, t test, $df = 4$), including a 3' UTR insertion in *SLC2A1* and intronic insertions in *PHGDH* and *EFHD1* (Figure S5). These examples resemble those seen in other cancers in which intragenic L1 insertions in tumors coincided with reduced host gene expression (Lee et al., 2012). To our knowledge, downregulation of *SLC2A1*, *PHGDH*, or *EFHD1* has not previously been associated with cancer.

The remaining tumor-specific L1 insertion occurred in donor 47 and was associated with activation of the transcriptional

repressor suppression of tumorigenicity 18 (*ST18*), a member of the MYT1 zinc-finger transcription factor family (Yee and Yu, 1998). Contrasting reports depict *ST18* as a tumor suppressor and as an oncogene in different cancers (Jandrig et al., 2004; Steinbach et al., 2006). *ST18* is, however, very poorly expressed in liver (Jandrig et al., 2004), making it unlikely to act as a tumor suppressor in this context. Ectopic host gene expression was an unusual consequence of an L1 insertion given that these events are usually repressive (Han et al., 2004). As such, we hypothesized that *ST18* was a candidate liver oncogene activated via an unknown mechanism triggered by an intronic L1 insertion.

Initial data from RC-seq indicated a heavily 5' truncated, 410 bp L1-Ta arranged antisense to *ST18* (Figure 6A). The integration site incorporated a 17 nt TSD, a degenerate L1 endonuclease motif (GC/AAAA), and a 112 bp 5' inversion of the L1 (Figure 6B), consistent with twin priming (Ostertag and Kazanian, 2001b). We then confirmed these features by PCR amplification and capillary sequencing of the L1 5' and 3' junctions, indicating a tumor-specific L1 insertion (Figure 6C). PCR on DNA extracted from three distinct biopsies taken from the same tumor detected

the L1 in all three regions, suggesting clonal amplification of tumor cells with the L1 mutant *ST18*.

As noted above, qRT-PCR indicated that *ST18* expression was significantly increased in tumor versus adjacent nontumor liver ($p < 0.005$, t test, $df = 4$) (Figure 6D). To corroborate this result, we performed an immunoblot and immunohistochemistry with an anti-*ST18* antibody and found *ST18* was indeed ectopically expressed in donor 47 tumor (Figures 6E and 6F). Chromosomal gain and regional copy number variation (CNV) have previously been reported for chromosome 8q, the genomic region containing *ST18* (Guichard et al., 2012). However, quantitative real-time PCR on gDNA indicated no *ST18* CNV in donor 47 tumor. Thus, tumor cells containing the *ST18* L1 mutation were clonally amplified without CNV of the *ST18* locus, followed by *ST18* transcriptional activation.

In response, we predicted that *ST18* was activated by insertional mutagenesis of a *cis*-regulatory element proximal to the L1. In silico analysis of the L1 integration site indicated that it bisected a palindromic motif containing two 8 bp units differing by one nucleotide and separated by 3 bp (Figure 6G). The probability of a random insertion in this motif, even allowing for a mismatch in the palindrome and a generous gap of ≤ 11 bp, was less than 1/1,000 (permutation test). Intriguingly, each unit was only one nucleotide different to a strong MYT1 binding motif found in the enhancer of *PIT1* (Rhodes et al., 1993). Previous experiments predicted that these units would bind MYT1 with reduced efficiency (Jiang et al., 1996), though transcription factors incorporating two zinc-finger domains, as for MYT1, are known to greatly gain efficiency through binding tandem DNA motifs (Yee and Yu, 1998). The putative MYT1 binding site was proximal to a strong binding site for CEBPA, a transcription factor enriched in liver and known to bind active enhancers (Johnson et al., 1987).

Based on this computational analysis, we predicted that the L1 bisected an enhancer normally bound to the zinc fingers of the *ST18* MYT1 domain. To test this experimentally, we performed chromatin immunoprecipitation (ChIP) of DNA bound to the *ST18* protein in Huh7 cells, followed by PCR amplification of the putative *ST18* enhancer. This assay confirmed that, absent an L1 insertion, *ST18* was preferentially bound to its own enhancer ($p < 0.0004$, t test, $df = 4$) (Figure 6H). An L1 insertion in the *ST18* binding site would reasonably be expected to displace this repressive mark from the enhancer. Thus, we experimentally validated a model of *ST18* activation in which a negative feedback loop was interrupted by a tumor-specific L1 insertion.

Finally, in view of the clonal amplification of tumor cells containing ectopically expressed *ST18*, we engaged complementary in vitro and in vivo experimental models to assess *ST18* oncogenic function in HCC. Although *ST18* is poorly expressed in liver, we found it to be abundant in several liver cancer cell lines (Figure S6A). We then determined the frequency of *ST18* CNV in an *Mdr2*^{-/-} mouse model of inflammation-driven HCC. TaqMan quantitative real-time PCR detected *ST18* amplification in 4/23 *Mdr2*^{-/-} HCC nodules and no deletions (Table S7). A disproportionately high percentage of advanced tumors (75%) presented *ST18* amplification. *ST18* expression was also significantly higher in nodules with amplified *ST18* compared with

wild-type mouse liver ($p < 0.0001$, t test, $df = 19$) (Figure S6B). These experiments demonstrate concordance of frequent *ST18* amplification and upregulation in human and mouse models of HCC, results consistent with *ST18* functioning as a candidate liver oncogene.

DISCUSSION

The present study highlights endogenous L1-mediated retrotransposition in the germline and somatic cells of HCC patients. We report two archetypal mechanisms revealing *MCC* and *ST18* as HCC candidate genes. *MCC* is, for the many reasons highlighted above, a highly plausible liver tumor suppressor. Four out of 19 individuals studied here, including two cases each of HBV and HCV infection, presented distinct germline L1 or *Alu* insertions contributing to *MCC* suppression in tumor and nontumor liver tissue. Strong upregulation of CTNNB1 in all four donors was consistent with prior observations that CTNNB1 is inhibited by *MCC* (Fukuyama et al., 2008). It is also interesting that *MCC*-L1- α was homozygous in donor 95, and therefore, *MCC* was almost certainly downregulated in the liver of this patient prior to HBV infection, i.e., preceding viral challenge, cirrhosis, and tumorigenesis.

We also demonstrate that *MCC* transcriptional repression in all four affected donors was exclusive of *APC*. Mutated *APC* is common in colorectal cancer but rare in HCC (Guichard et al., 2012; Powell et al., 1992). Even in colon, *MCC* presents numerous properties of a tumor suppressor (Bouwmeester et al., 2004; Fukuyama et al., 2008; Kohonen-Corish et al., 2007; Matsumine et al., 1996). Indeed, a Sleeping Beauty transposon mutagenesis screen using a mouse model of colorectal cancer found specific mutations in *MCC* and *APC* at a 1:9 ratio (Starr et al., 2009). Very recently, exome resequencing identified sporadic *MCC* point mutations in HCC (Guichard et al., 2012). Thus, *MCC* has potential to act as a liver tumor suppressor independent of *APC*, and our results support this potentially pivotal line of enquiry.

Tumorigenic retrotransposition in somatic cells was first observed 20 years ago, coincidentally in the *APC* gene of an individual with colorectal cancer (Miki et al., 1992). High-throughput sequencing has since provided the means to test whether tumor-specific retrotransposition is a common feature of cancer. Our results indicate that L1 mobilization occurs in a minority of HCC tumors, adding to the list of epithelial cancers (lung, colon, ovarian, and prostate) known to support the phenomenon (Iskrow et al., 2010; Lee et al., 2012; Miki et al., 1992; Solyom et al., 2012). Although transformed tumor cells, including liver cancer cell lines, support frequent transgenic L1 mobilization (Moran et al., 1996), it is unknown whether endogenous L1 activation precedes neoplastic transformation in vivo. For this reason, it was interesting that L1 transcription was found in liver tissue adjacent to tumors, in addition to an example of somatic L1 mobilization. Finally, in a small cohort of tumor-specific L1 insertions, we identified mobilization via TPRT, twin priming, and a third mechanism resulting in a small deletion and no TSD, as reported elsewhere (Gilbert et al., 2002). These observations highlight the multiple routes by which L1 mobilization alters the tumor cell genome.

The results presented here corroborate recent data generated via whole-genome sequencing of other cancers. As in our study, Lee et al. (2012) described tumor-specific L1 insertions bearing the hallmark features of TPRT and also found intragenic L1 insertions in differentially expressed genes (Lee et al., 2012). One distinct feature of the current study is our discovery that germline L1 and *Alu* insertions significantly perturb expression of genes relevant to HCC. Another advance is our explanation for the occasional activation of host genes by tumor-specific L1 insertions, based on an example of an interrupted negative feedback loop. The method presented by Lee et al. (2012) is convenient inasmuch as existing whole-genome sequencing data can be re-analyzed to identify novel retrotransposon insertions. However, we generated similar results with per sample sequencing depth 1/12 that of Lee et al. (2012), suggesting RC-seq is more efficient for new studies specifically focused on retrotransposons.

L1-mediated insertional mutagenesis revealed *ST18* as a candidate oncogene in HCC. Numerous corroborating observations support this possibility, including (1) clonal amplification of tumor cells containing the L1 mutant *ST18*, (2) ectopic *ST18* transcription and translation in tumor not seen in adjacent nontumor liver or control liver, (3) consistent *ST18* expression in transformed liver cancer cell lines, (4) frequent amplification of *ST18* in HCC nodules taken from *Mdr2*^{-/-} mice, and (5) induction of *ST18* transcription in those animals. However, we do not make any conclusion regarding the function of *ST18* as a tumor suppressor or oncogene outside of the liver and draw attention in this matter to *KLF4*, a transcriptional repressor known to function as a tumor suppressor and as an oncogene, depending on context (Rowland et al., 2005).

Overall, our results illustrate the confluence of multiple genetic aberrations in HCC, where inherited and de novo retrotransposition events form part of a wider mutational landscape. The experiments presented here and elsewhere suggest L1 activity varies substantially between individuals and cancer types (Iskrow et al., 2010; Lee et al., 2012; Solyom et al., 2012). It remains to be proven whether this phenomenon correlates with prognosis, is useful in a diagnostic capacity, or can be subjected to exogenous interference in vivo. Nonetheless, we can conclude that L1-mediated retrotransposition is a potentially crucial source of mutations that can reduce the tumor suppressive capacity of somatic cells in HCC.

EXPERIMENTAL PROCEDURES

Full protocols can be found in the Extended Experimental Procedures.

Samples

Tumor and nontumor liver tissues from 19 HCC patients with a confirmed HBV or HCV infection were provided by the Centre Hépatobiliaire, Paul-Brousse Hospital. DNA and RNA were extracted with a DNeasy Blood and Tissue Kit (QIAGEN, Hilden, Germany) and a mirVana miRNA Isolation Kit (Life Technologies, Carlsbad, CA, USA), respectively. Control liver samples from five donors were provided by the Edinburgh Sudden Death Brain and Tissue Bank. DNA and RNA were isolated through standard phenol-chloroform extraction and RNA-Bee RNA isolation reagent (Tel-Test), respectively. Samples were analyzed with approval from the French Institute of Medical Research and Health (Ref: 11-047), the East of Scotland Research Ethics Service (Ref: LR/11/ES/0022), and the Mater Health Services Human Research Ethics Committee (Ref: 1915A).

RC-Seq Library Preparation, Sequencing, and Analysis

Multiplexed DNA sequencing libraries were constructed for HCC tumor and nontumor samples using a paired-end Illumina TruSeq Kit with substantial modifications. Briefly, 1 µg of sonicated DNA size selected for an insert size of 200–250 bp was used for each library and amplified by six cycles of ligation-mediated PCR (LM-PCR). Libraries were then pooled in groups of 4 to 6 and hybridized to an updated custom Roche NimbleGen sequence capture array comprising oligos tiling the 5' and 3' termini of active human retrotransposon consensus sequences (Figure 1; Table S1). Libraries were again amplified by six cycles of LM-PCR and sequenced on an Illumina HiSeq2000. After quality filtering, each read pair was assembled into a contig, aided by 2 × 150-mer sequencing and a 220 nt insert size. Read contigs were then aligned to retrotransposon consensus sequences to determine their retrotransposon donor family, aligned to the human reference genome (hg19) to determine their genomic position, and finally formed into clusters.

PCR Validation

Germline retrotransposon insertions detected by RC-seq were first validated by a standard empty site/filled site PCR assay and then, if unsuccessful, with PCR targeting an insertion site 5' or 3' end. Tumor-specific insertions were characterized with a similar strategy but also incorporated 5' and 3' end capillary sequencing. All validation was performed on nonamplified DNA stored and handled separately from postamplification RC-seq products. Primers were designed using custom Python scripts and Primer3.

qRT-PCR

Complementary DNA was synthesized from total RNA using random hexamers, except for L1 analyses, where a specific sense L1 primer was used. qRT-PCR was performed using a LightCycler 480 (Roche, Indianapolis, IN, USA), and values were normalized to TATA-binding protein (*TBP*). For primer sequences, see Table S8.

ACCESSION NUMBERS

RC-seq FASTQ files were deposited in the European Nucleotide Archive (ERP001476).

SUPPLEMENTAL INFORMATION

Supplemental Information includes Extended Experimental Procedures, six figures, and eight tables and can be found with this article online at <http://dx.doi.org/10.1016/j.cell.2013.02.032>.

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